

REGENERATION IN THE AFRICAN LUNGFISH, *PROTOPTERUS*. III. REGENERATION DURING FASTING AND ESTIVATION

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Problems related to growth are among the most interesting in contemporary biology. Of unique interest are those special systems which can be isolated from, or juxtaposed with, normal somatic growth and maintenance. Limb regeneration in lower vertebrates is such a system, providing the opportunity to observe the reorganization of a replacement structure whose growth rate and degree of differentiation temporarily differ from those of the body as a whole.

The African lungfish, *Protopterus*, is especially suited to such experiments because growth and regression are already a part of its annual life cycle. Found in many of the river systems and lakes of tropical Africa, lungfish lead an aquatic life in flooded swamplands during the rainy season but survive the dry season in a state of metabolic torpor known as estivation. For five to seven months they remain buried in drying earth, unable to eat or excrete, protected from desiccation by a cocoon of dried mucus, and released only when the rains come again.

Estivation effects significant loss of body weight and shrinkage of trunk and appendages. Yet when not estivating, these fish are capable of prodigious growth and of full limb and tail regeneration (Conant, 1970). The experiments reported here counterpose these capabilities of regression and growth. Estivation has been induced in regenerating animals in order to investigate what influence, if any, the depressive effects of estivation may have on regeneration. Initial studies (Conant, 1970) have shown that very little regeneration occurred if estivation was induced prior to blastema formation. This paper will report on the effects of estivation begun during the growth phase of regeneration and will compare those results with observations on regeneration during periods of starvation.

MATERIAL AND METHODS

The fish used were 9 individuals of *Protopterus annectens* Owen and 2 of *P. aethiopicus* Heckel, ranging in weight from 51 to 382 grams after amputation. Each was maintained in a separate aquarium at $21^{\circ} \text{C} \pm 3^{\circ} \text{C}$ and fed beef strips twice a week. Portions of pectoral and pelvic limbs and up to 8 cm of tail were removed during anesthesia with 1:900 ethyl m-aminobenzoate methane-sulfonate: water, and regeneration was followed for up to 12 weeks in the water environment before estivation was induced. Over the course of three years, 15 experiments were completed in which the fish were induced to estivate while in the process of limb and tail regeneration. They were kept in the mud 3 months or more, a time period adequate for complete regeneration in the non-estivating, well-fed controls. The stage of the regenerating structures at the beginning of estivation varied with

different fish from 7 days to more than 2 months after amputation. At the end of an average time of 129 days (92–147 days), the fish were excavated, weighed and measured, and returned to water where growth resumed under conditions of adequate food and normal day-night illumination. Two additional fish died in my attempt to force estivation, and two died in the mud.

Estivation was induced by introducing the fish into a deep bucket of water and mud and gradually withdrawing the water over a period of 6–8 days. Grass seed

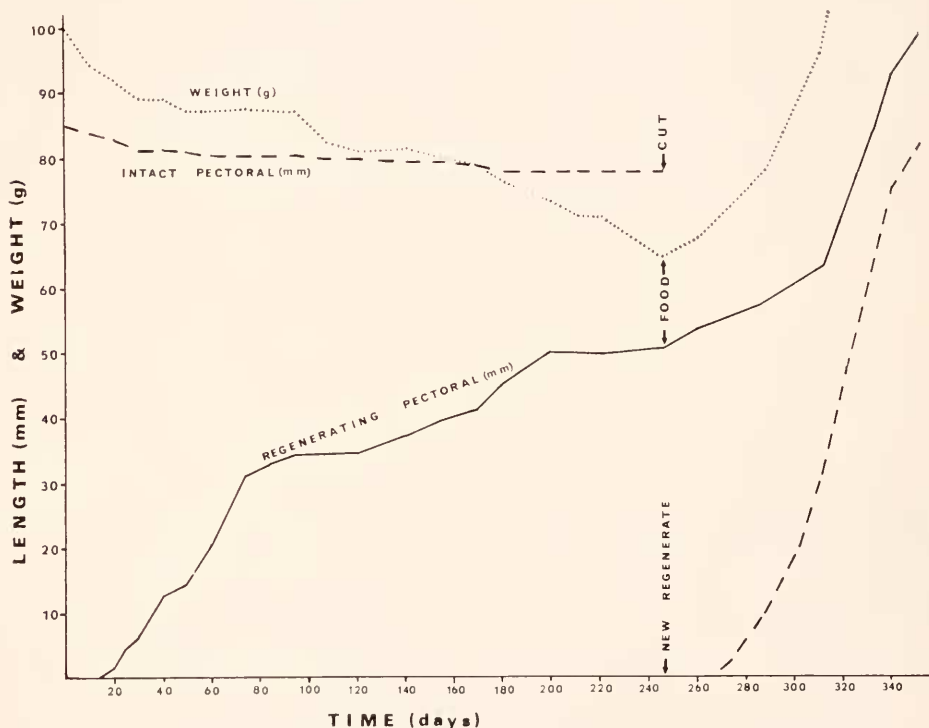


FIGURE 1. Changes in limb length (in mm) and body weight (in g) of fish #18, *Protopterus acthiopicus*, fasted for 246 days and then fed. Growth of regenerating pectoral limb measured under conditions of both fasting and feeding. Shortening of intact contralateral pectoral limb measured during fasting; then that limb was removed, feeding was resumed, and measurements were taken of the new regenerate.

planted near the respiratory channel enhanced dehydration and helped prevent localized cracking as the mud dried.

Three fish were starved for 212, 246, and 308 days, respectively, following the amputation of one pectoral limb 3 mm from the body. Regular records were kept of weight, snout-tail length, and the length of the two pectoral limbs. In the case of fish #12, the pectoral limb left intact had itself regenerated 8 months prior to the beginning of the experiment. The control limb on the other two fish (#18 and #28) had not regenerated in at least the three previous years. At the end of the fasting period, the control limb was removed and feeding resumed.

Fish #28 developed fungus infection and died, but growth was followed in the other two.

OBSERVATIONS

Regeneration during fasting

A regenerating appendage in a well-fed lungfish kept in water passes through a period of wound healing (1–2 days), a latent period of 2–3 weeks, and then periods of first accelerating and then decreasing growth, with replacement complete after 3–4 months (Conant, 1970). The regenerating pectoral limb in the three fasting fish showed healing and latent periods roughly similar to those of fish that were being fed, but elongation during the growth phase was considerably attenuated.

Figure 1 summarizes changes in limb length and body weight observed in fish #18 during and after a starvation period of 246 days. Data from the other two fasted fish closely resembled these data from #18. At the end of 3 months, by

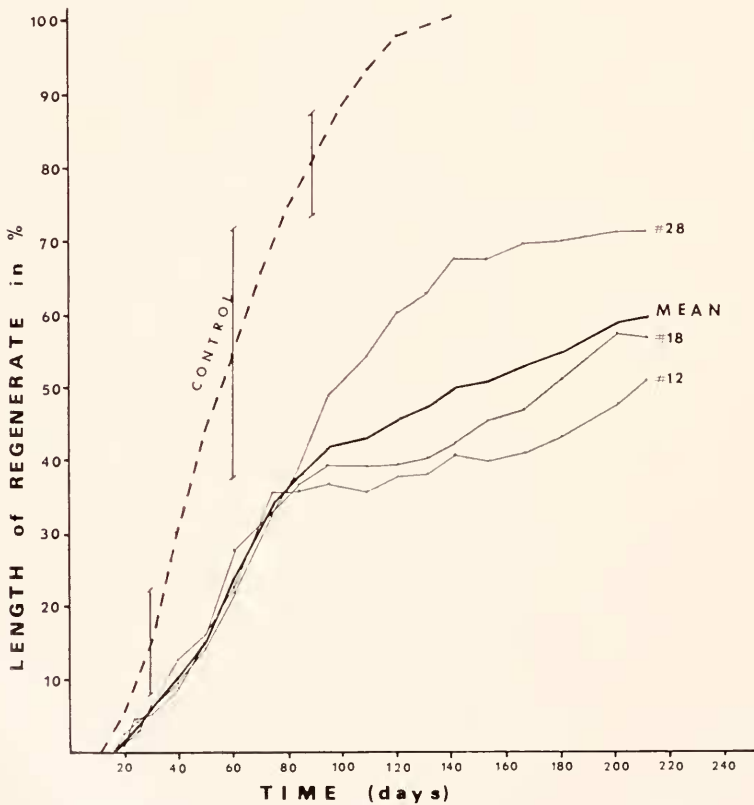


FIGURE 2. Growth curves of the regenerating pectoral limb of three fasting fish and their mean, compared to a curve representing the mean pectoral regeneration in 12 control fish kept in water with adequate food. All curves are expressed in terms of per cent replacement of the amputated segment over time. Vertical lines intersecting the control curve at days 30, 60, and 90 represent standard error.

which time a pectoral regenerate would have been totally restored under well-fed circumstances, the regenerating pectoral of the fasting fish attained a length of 30–35 mm, barely a third of the 87 mm removed. By 8 months, the new limb measured approximately 50 mm, or about 60% of its original length. Upon resumption of feeding, substantial weight gain accompanied accelerating growth in the original regenerate, and $3\frac{1}{2}$ months later the limb was 100% restored.

Figure 2 depicts the growth curves of regenerating pectoral limbs in all three fasting fish compared to the mean rate of pectoral regeneration in 12 control fish kept in water and fed regularly. Statistical comparisons of these growth rates were made by two methods. A comparison of the extent of limb replacement at 10-day time intervals showed significant differences between the fasting and control fish with probabilities of identity being less than 0.05 from day 40 onward, less than

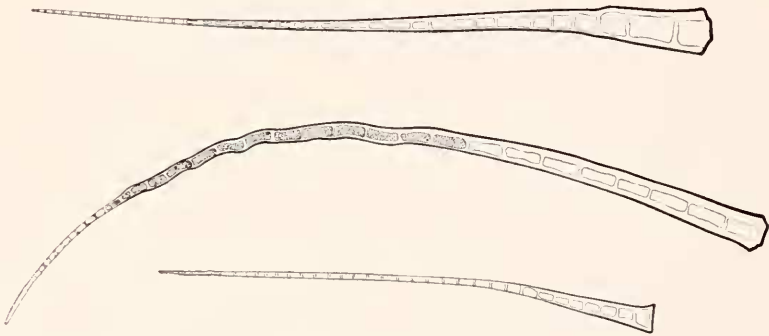


FIGURE 3. Tracing of three pectoral limbs taken from fish #28 before and after 212 days' fast. The uppermost limb is the left pectoral, recently regenerated, removed on day 1 of the experiment. The middle limb is the right pectoral, removed on day 212, terminating fast. Note its deformation in the course of atrophy, the axial segments most involved having been stippled. The lowermost limb is the left pectoral regenerate, grown during the fasting period and removed on day 215 when the animal died of fungus infection. Note the straight axis of both the new regenerate and the limb removed as the experiment began. Note also that the terminal segments are less distinct in the lower two limbs.

0.001 from day 70 onward. The second approach involved examination of the slope of the growth curves. Using the arc tangent of the increment of growth added during each successive 10-day time period, the curves were expressed in degrees of slope. Comparison of the slope angles for the first 130 days shows significant differences between the experimental and the control rates with *P* values of 0.05 or less from day 30 to day 50 and from day 90 to day 120.

The loss of body mass during starvation was manifest in several ways. Weights decreased steadily: the three fish lost 40%, 36%, and 26% of their initial weights in the 7–10 months' fast, the fish showing the greatest loss (fish #28) dying of fungus infection shortly after feeding was resumed. At the end of these prolonged fasts, the atrophy of the head musculature was clearly visible in fish #18 and #28, and the skin seemed to "fit loosely." Axial shrinkage amounted to 8% or less of the original body length; girth measurements might have been more revealing.

The intact pectoral limb also shortened during the fasting period. In fish #18 and #28, this "control" limb was 7 mm shorter after 7–8 months, a loss of

about 10%. In fish #12, the intact limb had itself recently regenerated; it shortened during fasting by 19 mm, about 20% of its length at the beginning of the experiment. In each case, most of the loss in length occurred in the early weeks, and after several months the straight axis of the shortened limb was deformed by wavy irregularities (Fig. 3).

In order to investigate whether or not the prolonged fast exerted a lasting depressant effect on growth, the intact pectoral limb was removed at the end of the fasting period and the regrowth of its replacement was observed under conditions of feeding (Fig. 1). Statistical comparisons of both the extent of growth and the degree of slope of the new post-fast regenerate vs. the control curve (Fig. 2) showed no significant differences.

TABLE I
*Summary of regeneration/estivation results, ranked in order
of per cent replacement at the start of estivation*

Fish # (weight)	Induced to estivate on day # after amputation	Length of re- generate at beginning of estivation in % replacement:			Mean %	Mean in mm	Total days in estiv. (weight at end)	Length of re- generate at end of estivation in % replacement:			Mean %	Mean in mm
		Pec- toral	Pel- vic	Tail				Pec- toral	Pel- vic	Tail		
#28,a (202 g)	7	0	0	0	—	—	129 (150 g)	4.7	2.8	0	2.5	1.5
#29,a (132 g)	7	0	0	0	—	—	129 (100 g)	3.6	5.7	0	3.1	1.3
#14 (286 g)	19	2	0	0	0.7	0.5	146 (264 g)	5.4	12.2	0	5.9	3.3
#07 (110 g)	18	1.7	3.3	0	1.7	0.8	147 (100 g)	6.9	11.1	12.3	10.1	5.7
#12 (382 g)	21	4	2.8	1.1	2.6	1.3	136 (358 g)	23.3	17.1	32.6	24.3	15.8
#27,a (203 g)	18	5.7	12.5	6.3	8.2	3.0	92 (194 g)	11.4	32.5	18.8	20.9	7.3
#18 (100 g)	18	12.5	16.7	1.1	10.1	3.7	92 (98 g)	17.5	22.2	20.7	20.1	7.5
#28,b (169 g)	34	19.0	18.2	3.9	13.7	8.2	140 (135 g)	22.2	20.9	9.2	17.4	10.5
#29,b (102 g)	29	19.5	15.3	10.0	14.9	5.8	140 (93 g)	21.9	27.8	40.0	29.9	11.7
#21,b (235 g)	39	22.5	33.3	7.1	20.9	12.7	136 (180 g)	20.0	38.1	21.2	26.4	16.7
#21,a (177 g)	42	40.5	40.0	10.0	30.2	12.3	130 (158 g)	29.9	30.0	16.7	25.5	10.3
#16 (51 g)	72	38.5 (28.5)	36.6	33.3	34.2	13.2	129 (49 g)	35.9 (14.3)	36.6	44.4	32.8	14.0
#22 (130 g)	88	40.0	72.4	21.3	44.6	26.3	129 (124 g)	30.0	55.2	24.0	36.4	19.3
#27,b (166 g)	87	90.0	60.0	37.7	62.6	37.7	133 (138 g)	73.3	40.0	35.0	49.4	30.3
#19 (143 g)	71	96.6	79.4	47.4	74.5	42.7	133 (130 g)	75.9	48.9	41.0	55.3	32.5

Regeneration during estivation

The data from the regeneration/estivation experiments are presented in Table I. The data are ranked in terms of the average amount of regeneration in the three appendages compared to the average amount removed ("% regrowth") at the time estivation was induced. The mean length of the regenerates in millimeters is also recorded, as is the length of the estivating period and the weight before and after estivation. It should be remembered that a well-fed control fish in water would show virtually 100% replacement of parts removed during the 3-5 months time period of the estivation experiments.

These data indicate that estivation sharply curtailed but did not stop regeneration when the fish appendages were in the latent or early growth phase of regeneration at the time of entry into the mud. Estivation, however, not only inhibited further growth of longer regenerates but actually effected a loss in length.

Fish induced to estivate at the end of week one of the latent phase showed growth of only a few millimeters (2-3%) at the end of 129 days in the mud, a point which

would have been reached within 14 days had the fish remained in water. Regenerates which were somewhat farther along at the time of estivation increased an additional 5–15% in length during the 3–5 months in the mud, although there was such individual variability that no direct linear relationship was found between length before and after estivation. When the regenerating structures averaged more than about 1/3 replacement at the outset, however, estivation exerted still greater limitation such that, in a series of increasingly mature regenerates, first the pectoral, then the pelvic, and finally the tail regenerates shortened in length while in the mud. All these regenerates, no matter what their stage and whether

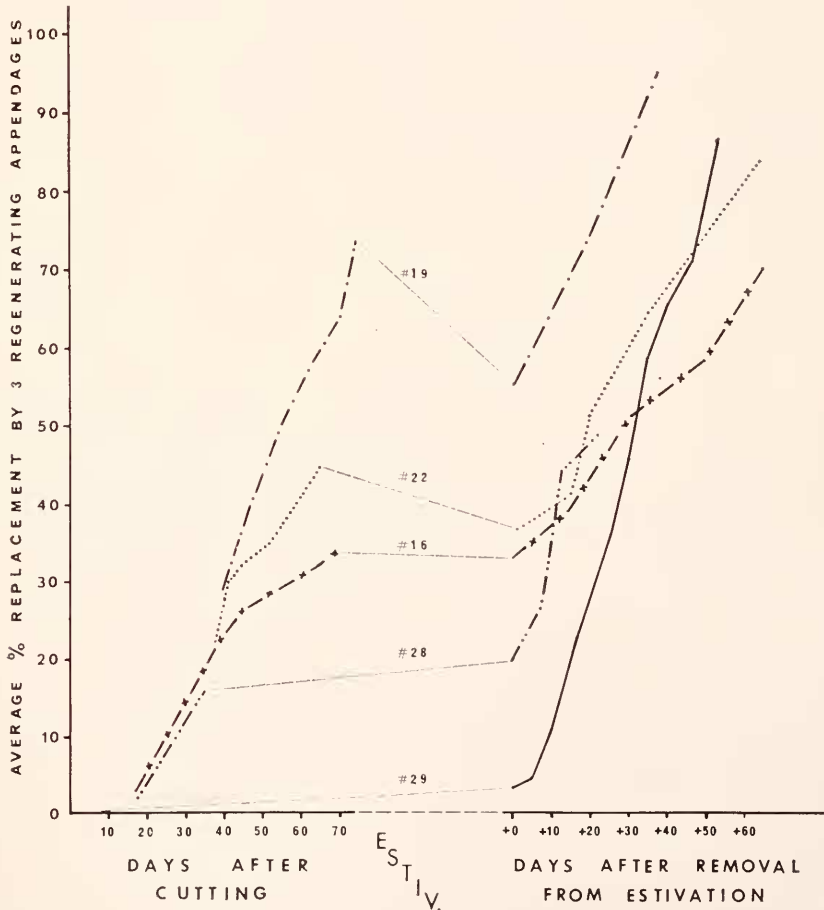


FIGURE 4. Representative growth curves of five lungfish before and after estivation. The curves at the left are segments of the growth curves following amputation, drawn to show successively longer regenerates. The dashed lines in the middle represent 3–5 months in estivation, not drawn to scale. The curves at the right represent growth data after the fish were returned again to water. Note the limited growth during estivation in the earlier stages of regeneration, the loss of length during estivation by limbs in the later stages of regeneration, and the resumption of growth after estivation was terminated.

they had grown or shrunk during estivation, resumed growth when the fish were returned to water and feeding was reestablished (Fig. 4).

There were characteristic differences in growth patterns among the two limbs and tail. Figure 5 expresses growth before and after estivation in absolute length (mm) in bar-graph form, ranking each experiment in terms of average length of the three regenerates at the time of induced estivation. This graph, and the

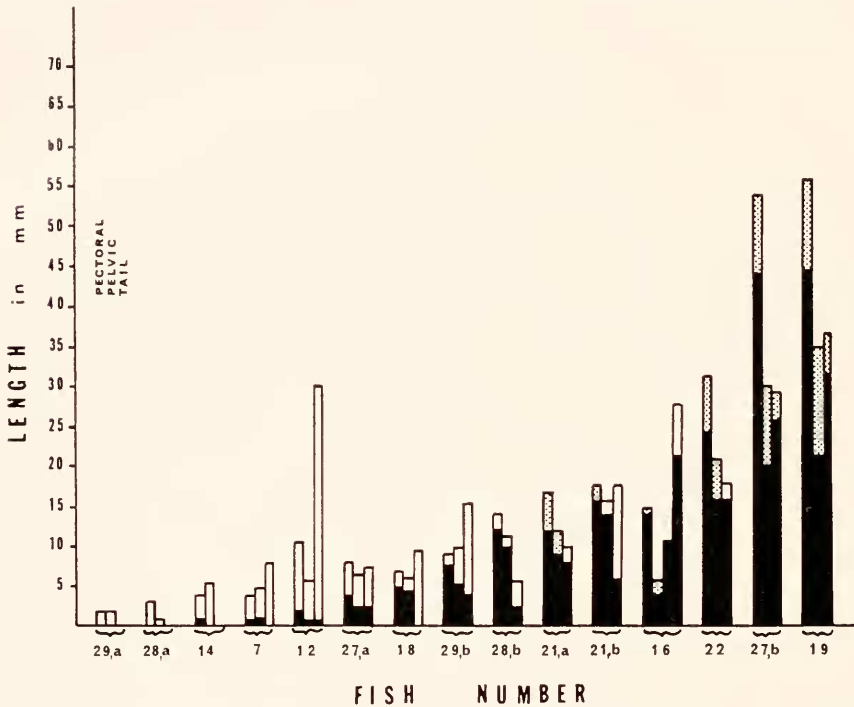


FIGURE 5. Regeneration/estivation data from 15 experiments, using measurements in absolute length (mm) and ranked in order of increasing length at the time of estivation. Each trio of bars represents measurements of, from left to right, the pectoral limb, the pelvic limb, and the tail. Data from fish #16 includes a second pectoral limb at the left. Dark area represents extent of growth at the beginning of estivation; white area represents increment of additional growth during estivation; stippled area represents the extent of shrinkage during estivation, *i.e.*, the highest point of the stippled bar denotes the length when estivation was induced, the stippled area indicates decrement lost during estivation, and the height of the blackened section defines the length of the appendage when estivation was terminated.

comparable information in Table I, shows that the three appendages responded somewhat differently to amputation and estivation. Typically, the pectoral limb breaks the lag phase first, grows the fastest, and completes regeneration before the others, consistent with previous observations that rapid growth compensates extensive limb amputations (Conant, 1970). In absolute measurement, the pelvic limb responds to amputation a little more slowly, and, until later in the growth process, the tail grows at the slowest rate of all. Hence, in Figure 5, the blackened

bars representing the extent of regeneration when estivation was induced usually show that the greatest length was attained by the pectoral limb.

This characteristic "lead" of the pectoral regenerate in the water environment was not maintained during estivation. In Figure 5, the white bars represent growth during estivation. In the majority of cases, the incremental growth of the pectoral limb while in the mud was less than that of the pelvic limb or tail, and of all structures, the tail seemed the least affected by estivation. The regenerating tail, for example, frequently added length while the limbs showed gain or even loss, and in one case (fish #12) where cocoon formation was incomplete, the tail regenerate added nearly 3 cm in 136 days in the mud. The pectoral limb, on the

TABLE II
Regeneration during short-term estivation

Fish #:	Fish #21 (1971)	Fish #26	Fish #21 (1972)	Fish #22	Fish #12
Pre-estivation:					
% regrowth, pectoral	16.7%	41.3%	59.5%	43.1%	86.7%
% regrowth, pectoral				53.8%	54.5%
% regrowth, pelvic	16.7%	27.3%	7.4%	36.7%	41.2%
% regrowth, pelvic				32.4%	
% regrowth, tail	2.5%	34.3%	37.7%		64.5%
Mean of all regenerates in % regrowth	11.9%	34.3%	34.9%	41.5%	61.7%
Mean of all regenerates in mm	4.7 mm	21.7 mm	20.3 mm	19.8 mm	41.3 mm
Days in estivation:	21	21	27	27	27
Condition of cocoon:	unformed	unformed	dry	dry	dry
Post-estivation:					
% regrowth, pectoral	23.8%	43.8%	43.2%	34.5%	70.7%
% regrowth, pectoral				41.0%	48.1%
% regrowth, pelvic	30.0%	30.9%	18.5%	33.3%	38.2%
% regrowth, pelvic				26.0%	
% regrowth, tail	5.0%	36.0%	41.3%		77.8%
Mean of all regenerates in % regrowth	19.6%	36.9%	34.3%	33.7%	58.7%
Mean of all regenerates in mm	7.7 mm	23.3 mm	18.0 mm	16.5 mm	37.3 mm

other hand, appeared to be the most labile, the first to start growth, but the first to show inhibition. In Figure 5, the stippled sections of the bars represent the extent of loss of length during estivation, and it can be seen that the pectoral limb was the first, the tail last, to undergo shrinkage.

One experiment tested the effects of estivation on an induced pectoral branch. Accessory structures can be elicited in lungfish by removing from the limb a wedge of tissue which includes some axial endoskeleton (Conant, 1972). Fish #14 had been operated on in this fashion, and by the time of estivation (19 days), the cut had filled in and a 1 mm projection had formed in its place. After 146 days in the mud, the projection had neither grown nor shrunk, but it sprouted rapidly once the fish had been returned to water and to feeding. This was a case in which a new structure was held in abeyance by estivation but was not inhibited when conditions improved.

Table II lists the data from five fish (17 appendages, total) induced to estivate when the appendages were in varying states of regeneration and which were excavated after 3–4 weeks instead of 3–5 months. Because of differences in the number of appendages cut, the format of Table II is reversed from that used in Table I. It can be seen that fish in earlier stages of regeneration and those with less complete cocoon formation were less affected by estivation than those induced to estivate at a later stage or whose cocoons were fully formed.

DISCUSSION

Many lower vertebrates can grow and/or regenerate despite adverse conditions. As early as 1909 Ellis showed that fasting tadpoles could regenerate at the same rate and to the same degree as well-fed counterparts, and as recently as 1970 Hui and Smith reported that *Ambystoma* larvae unfed for 10 weeks regenerated hind limbs fully as rapidly as controls despite a 50% loss of body weight.

Lungfish would seem to be exceptions to this general rule. They routinely encounter environmental adversities, and in the course of evolution have developed numerous adaptations to minimize the effects. Sluggish as well as poikilothermic, they are capable of prolonged fasting and may experience 6 months without food in estivation as an annual event. Smith (1930, 1935a), in work later expanded by Janssens (1964), examined the metabolism of two specimens of *P. aethiopicus* starved until death and found several metabolic adjustments. Levels of body carbohydrate and enzyme titers were found to differ significantly in control, starving, and estivating animals. Smith further reported a persistent decrease in O_2 consumption roughly following a logarithmic curve. Using his figures, the fish described here would be consuming only about 20% as much O_2 after 7–8 months' fast as normally. Since protein degradation, an inefficient energy source, accounts for 50% or more of the metabolic energy, the fasting or estivating lungfish obtains only about 1.6 cal for every gram of body weight lost (Smith, 1930). At the end of 250 days, the two fish in Smith's experiments had lost approximately 9% and 18% of their original body weight, smaller figures than those reported here in my specimens. His experiments were run at 20° C. One final observation supporting the notion of the conservation of body energy during fasting concerns the animal's activity. Normal well-fed lungfish show limited nocturnal movement in the form of occasional episodes of swimming alternating with quiet periods. These movements can be detected by electrodes in the water and noted on a chart recorder. After 2 months of fasting, however, all such movements had been abolished, and, except for respiration, the fish remained apparently motionless for days at a time.

In the light of this inactivity during fasting and the enforced immobility of estivation, one wonders if "disuse atrophy" might play a role in the loss of body mass and in the retardation of regeneration found under both conditions. Inasmuch as structural proteins are used as an energy source, it would be difficult to differentiate what proportion of limb or tail regression could be attributed to metabolic degradation to counteract caloric deficiency and what proportion to simple disuse. Denervation experiments in combination with fasting or estivation might provide some useful clues.

In any case, considering these multiple responses to starvation, it may not be

surprising that the lungfish limb regenerated at a significantly lower rate when food was withheld. When feeding was resumed, the growth rate of this regenerating limb increased, consistent with Smith's observation (1935b) that feeding-metabolism levels of O_2 consumption were rapidly reinstated once the fast was broken. Unlike Buchanan's report on anurans (1938), there was no evidence of compensatory increase in growth rate after the adverse conditions were relieved. This might be explained by Smith's further observation (1935b) that a maximum level of O_2 consumption was reached in lungfish beyond which ever-increasing amounts of food had no effect.

The regression of the intact pectoral limb during fasting took place at the same time that the new regenerate had begun to grow. Over the total period without food, the intact control limbs of fish #18 and #28 lost 10.6% and 7.5% of their original length and the intact but recently regenerated control limb of fish #12 lost 20% in length. This atrophy of the unamputated limb paralleled shrinkage of the axial snout-tail length by about 8% and losses of body weight in the 3 fish of 26-40% such that the regeneration of the amputated pectoral, albeit limited, could be seen as net growth taking place despite the overall tendency towards tissue reduction. The same can be said for those tissues which added mass during estivation despite a general loss of body mass in the time in the mud.

Estivation imposes even greater stringencies on the animal than fasting. Although cessation of feeding is an aspect of estivation, additional elements are involved such as immobility, cocoon formation, reduced heart rate and O_2 consumption, numerous biochemical and enzymatic adjustments (Smith, 1930; Janssens, 1964), virtual cessation of erythropoiesis (Jordan and Speidel, 1931), changes in endocrine activity (Godet, 1959, 1962; Godet, Michel, and Dupé, 1964; Leloup, 1958), and, according to Swan, Jenkins and Knox (1969), the possible production of a neurohumoral antimetabolite.

Reminiscent of the gross results of fasting, these internal changes of the lungfish in the mud are reflected in gross changes, most clearly in overall weight loss and in modification of body form. Estivation of only a few months in drying conditions results in the loss of up to 27% of the body weight (Smith, 1930; Lüling, 1961; Table I here). Further, during estivation, the tail tip becomes noticeably blunter as the axis shortens and all limbs lose some of their length. Blanc, d'Aubenton and Plessis (1956) reported that after 28 weeks of estivation, one fish had lost 57 g out of 345 g originally, was 35 mm shorter than the original 400 mm snout-tail length, had pectoral limbs only two-thirds their initial length, and pelvic limbs shortened by over 10%. In field observations in the Congo, Poll (1938) observed that estivating individuals of *Protopterus* excavated near the Lualaba had limbs reduced to as little as a third of the length of those belonging to active fish taken from open water. My own measurements of non-regenerating limbs show reductions in length of 6-11% in the pelvic and 19-35% in the pectoral limbs resulting from estivation.

From the experiments reported here, appendages which had regenerated about two-fifths or more of the amputated portion responded to estivation like whole limbs in that their lengths were decreased rather than increased during estivation. In light of the greater vulnerability of the recently regenerated limb to reduction during fasting referred to earlier, it would be interesting to know whether limb

shortening during estivation is more pronounced in regenerates than in those limbs which had never regenerated. Not having reared any of my experimental fish from larvae, I do not know their full history and cannot make this comparison.

In these experiments, early stages of regeneration were observed under four different conditions, and the resumption of growth by older regenerates was observed under two. New regenerates were seen developing (1.) under control conditions of water and food, (2.) during fasting, (3.) after the fast had been broken, and (4.) after release from estivation. Regeneration in later stages of growth was followed after the termination of fasting and estivation.

Comparison of the growth curves of the early stages of new regenerates reveals basic similarities in lag phase, time of blastema formation, and early growth. With food, whether after fasting or after estivation, development of the new structure virtually paralleled that of the controls; during fasting, development was slower than in the controls but much closer to the control rate for the first 11 weeks than later (Fig. 2). This suggests that the initiation of growth and its early stages are not profoundly affected by existing or recently terminated adverse conditions.

Older regenerates, on the other hand, showed a two month lag in the resumption of growth after 8-10 months' fast, a condition in marked contrast with the normal growth of the newly-amputated regenerate. Likewise, there is some indication in Figure 4 that the older regenerates did not resume the normal growth rate as speedily after estivation as did those whose growth had been interrupted in earlier stages.

In short, the state of the regenerate, so important in the growth/regression response to estivation, appears to affect the recovery process as well. Comparative studies of vascularization, mitotic index, enzyme levels and innervation might help define the degree of this state-dependency.

It is tempting to consider estivation in relation to hibernation, estivation resembling hibernation in metabolic depression but not in response to lowered temperatures. Can experiments in estivation help separate out the simpler effects of temperature from those of metabolic austerity and immobility? Invertebrate regeneration during hibernation has been studied with segmented worms, although they defy generalization in that some groups can regenerate during diapause while others cannot (Saussay, 1966a, 1966b). Among higher vertebrates, Lyman and others have examined changes in cold-acclimated and hibernating rodents. During hibernation, cell division (Mayer and Bernick, 1958) and DNA synthesis (Manasek, Adelstein and Lyman, 1965) were sharply curtailed, hematopoietic organs were inactive (Lyman, Weiss, O'Brien and Barbeau, 1957), and even neoplastic transplants in the hamster cheek pouch did not grow (Lyman and Fawcett, 1954). Among Chiroptera, there have been observations on changes in osteocytic activity (Whalen, Krook and Nunez, 1972) and on the lack of healing during hibernation (R. J. Goss, Department of Biology, Brown University, personal communication).

These experiments with *Protopterus* suggest an equally profound depressant effect under estivating conditions, this despite temperatures of about 23° C in the laboratory and 10° C or more higher in field measurements of dry mud in East Africa (Swan and Hall, 1966). The only growth noted during estivation was of appendages in early stages of regeneration and even that was sharply curtailed.

The process of estivation involves several stages: progressive penetration to the

deeper layers of the mud and the consequent formation of the respiratory channel, assumption of the final estivating position, production of heavy mucus, and the drying of mucus in final cocoon formation. The dryness of the environment determines in large measure the pace of these steps, and in some natural settings such as parts of the Congo, at least one species, *P. dolloi*, remains active in moist mud all during the dry season with no cocoon formation whatsoever (Brien, 1958). Observations based on the mud and containers used in this laboratory established that by 3 weeks the animal had taken up the final deep position and was covered with heavy mucus and that by 4 weeks with daily deep withdrawal of water, the cocoon was complete.

It is important to know what fraction of the growth recorded after 3–5 months in the mud had in fact taken place in the first 3–4 weeks. Accordingly, 5 fish with appendages in varying stages of regeneration were induced to estivate as usual but were retrieved after 3–4 weeks (Table II). The results mirror those seen after extended estivation. The fish that showed the greatest growth in the series was the one whose regenerating appendages were in their earliest stages, averaging 11.9% or 4.7 mm, and whose cocoon was not yet fully formed. This animal showed an average additional increment of regeneration during the 3 weeks of 3 mm, or 7.7%. Another fish (#26) whose regeneration was farther along at the time of estivation (34.3%, or 21.7 mm average) and who was excavated at 3 weeks before the cocoon was complete, showed additional growth of but 1.6 mm on the average (2.6%). Still less growth, or even shrinkage, was found in fish whose regenerates were still more extensive and/or whose dry cocoon had fully formed.

When do the depressant effects of estivation become operative? There are several clues to suggest that the relevant metabolic and behavioral adaptations take effect almost immediately. The regeneration figures themselves are the first clue (Tables I and II; Fig. 1). If 3 weeks in the mud preceding cocoon formation were as metabolically "normal" as 3 weeks of fasting in water, then one might expect growth figures to be comparable. Yet the increments added during the process of estivation are even less than those added during fasting, and indeed may even be decrements, suggesting that the conservative influences of even pending estivation are felt from the beginning. Elements of lungfish behavior likewise support this view. Within hours of being introduced into the mud, the fish will assume the tail-over-eyes estivating position even though this immobility is periodically interrupted by episodes of burrowing and position change. The respiratory behavior also changes in most fish within the first day, from the semi-regular lung-filling associated with the aquatic environment to clusters of breaths separated by periods of 20–50 minutes below the surface.

Nevertheless, the growth in the first few weeks of the short-term experiments does constitute a significant fraction of changes noted after longer estivation periods in other fish. Indeed, it may be that some of the growth of the pre-cocoon estivating stages would later have been nullified by limb shortening during extended periods in the final estivating state. For this reason, the results support the notion that maximum metabolic depression accompanies full cocoon development.

In conclusion, the dual capacity of the lungfish both to resorb and regenerate, both to estivate and grow, provides the opportunity to study growth under special conditions. It is just one more example of the many interesting features found in the Dipnoi.

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SUMMARY

1. Limb and tail regeneration of *Protopterus annectens* and *P. acthiopicus* was observed under conditions of fasting and induced estivation.

2. Three fish were fasted for 212, 246, and 308 days, respectively. During this period, weight losses of 26–40% were recorded. A regenerating pectoral limb on each fish showed healing and latent phases comparable to well-fed controls, but growth itself was considerably slower and averaged only 50–60% replacement after several hundred days. The contralateral intact pectoral shrunk 7–20% during the same period and axial length shortened by about 8%. After feeding resumed, weight was rapidly regained, the old regenerating limb increased its growth rate, and a new pectoral regenerate grew normally.

3. Fifteen fish were induced to estivate after they had regenerated varying amounts of limb and tail tissue. Estivation sharply limited but did not halt further growth if the regenerate was in the latent or early growth stages, but it did stop growth or even cause shrinkage of more mature regenerates. The pectoral regenerate, first to grow after amputation, was also the first to be inhibited by estivation; the tail was the last to be affected.

4. Short-term estivation experiments revealed that the bulk of the growth took place in the 3–4 weeks preceding dry cocoon formation. It is concluded that deep estivation has an inhibitory effect on regeneration.

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